art will understand that all of such methods are applicable to whole antibodies and functional fragments thereof as well as to regions and functional domains other than the antigen binding variable region of antibodies. Moreover, the methods described herein are further applicable to molecules other than antibodies, variable regions and other antibody functional domains. Given the teachings of the invention, those skilled in the art will know how to apply the methods of simultaneously constructing hybrid molecules and maintaining or optimizing the binding affinity or catalytic activity of a target molecule, as well as how to apply the methods of optimizing the binding affinity or catalytic activity to a variety of different types and classes of polypeptides and proteins.

It is understood that modifications which do not substantially affect the activity of the various embodiments of this invention are also included within the definition of the invention provided herein. Accordingly, the following examples are intended to illustrate but not limit the present invention.

EXAMPLE I

Simultaneous Humanization and Affinity Maturation of an Anti-CD40 Antibody

This example shows the simultaneous humanization and affinity maturation of the murine mAb 40.2.220, directed against the CD40 receptor.

The CD40 receptor is a potential therapeutic target for several diseases. For example, the interaction of the CD40 receptor and its ligand, gp39, serves a critical role in both humoral and cell-mediated

immune responses (Foy et. al., (1996)). Immunological rejection of organs from genetically non-identical individuals, termed graft-versus-host-disease (GVHD), is mediated through T cell-dependent mechanisms. *In vivo* administration of an anti-gp39 mAb blocks GVHD in mice and inhibits many of the GVHD-associated phenomena (Durie et. al., 1994)), providing evidence that the CD40/gp39 interaction plays a critical role in the development of GVHD. More recently, inhibition of the CD40/gp39 interaction *in vivo* in hyperlipidemic mice fed a high cholesterol diet limited atherosclerosis, suggesting that CD40 signalling may also play a role in atherogenesis (Mach et. al., (1998)). In addition, the CD40 receptor is overexpressed on hematologic malignancies (Uckun et. al., 1990)) and certain carcinomas (Stamenkovic et. al., (1989)) and thus, may serve as a target for cytotoxic agents. An anti-CD40 single chain antibody-toxin fusion was cytotoxic against CD40-expressing malignant cells *in vitro* (Francisco et. al., (1995)) and was efficacious in treating human non-Hodgkin's lymphoma xenografted SCID mice (Francisco et. al., (1997)).

Codon-based mutagenesis (Glaser et. al., (1992)) was used to create libraries of LCDR3, HCDR3 and framework region variants of mAb 40.2.220 sequences. Libraries composed of framework region variants alone and in combination with HCDR3 variants and with HCDR3 and LCDR3 variants together were screened for high affinity variants. It was demonstrated that in combination higher affinity variants were obtained than those obtained when codon-based mutagenesis was applied independently thus showing (1) higher affinity variants that could only be obtained by the use of codon-based mutagenesis simultaneously on disparate regions of the mAb and (2)

the use of codon-based mutagenesis to uncover potential direct interactions between disparate regions of a mAb.

A vector for the production of a chimeric anti-CD40 murine mAb 40.2.220 was constructed. Based on the sequence of anti-CD40 murine mAb 40.2.220 (provided by Dr. D. Hollenbaugh, Bristol-Myers Squibb, Princeton, NJ) overlapping oligonucleotides encoding V_H and V_L (69-75 bases in length) were synthesized and purified. The variable H and L domains were synthesized separately by combining 25 pmol of each of the overlapping oligonucleotides with *Pfu* DNA polymerase (Stratagene) in a 50 μl PCR reaction consisting of 5 cycles of: denaturing at 94°C for 20 sec, annealing at 50°C for 30 sec, ramping to 72°C over 1 min, and maintaining at 72°C for 30 sec. Subsequently, the annealing temperature was increased to 55°C for 25 cycles. A reverse primer and a biotinylated forward primer were used to further amplify 1 μl of the fusion product in a 100 μl PCR reaction using the same program. The products were purified by agarose gel electrophoresis, electroeluted, and phosphorylated by T4 polynucleotide kinase (Boehringer Mannheim) and were then incubated with streptavidin magnetic beads (Boehringer Mannheim) in 5 mM Tris-Cl, pH 7.5, 0.5 mM EDTA, 1 M NaCl, and 0.05% Tween 20 for 15 min at 25°C. The beads were washed and the non-biotinylated, minus strand DNA was eluted by incubating with 0.15 M NaOH at 25°C for 10 min. Chimeric anti-CD40 Fab was synthesized in a modified M13IX104 phage vector (Kristensson et. al., 1995)), termed M13IX104CS, by hybridization mutagenesis (Rosok et. al., (1996); Kunkel, (1985)) using the V_H and V_L oligonucleotides in 3-fold molar excess of the uridinylated vector template. The M13IX104 vector was modified by replacing cysteine residues at the end of the kappa and γ1 constant regions with serine. The reaction